

Bulletin No. 21 from the Department of Surgery,  
University of Lund, S-221 85 LUND,  
1980

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## BLOOD FLOW IN EXPERIMENTAL TUMORS

With special reference to the effect  
of vasoactive drugs.

A study in the rat.



KAJ SUNDQVIST

Lund 1980



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med lic., Vd.

Akademisk avhandling  
som för avläggande av medicine doktorsexamen  
vid Medicinska Fakulteten vid Universitetet i Lund  
kommer att offentligen försvaras å Föreläsningssal 3,  
Centralblocket, Lasarettet i Lund,  
tisdagen den 13 maj 1980 kl 09.15.

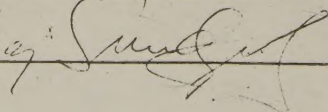


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| Organisation<br><b>LUND UNIVERSITY</b>                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                             | Document name<br><b>DOCTORAL DISSERTATION</b> |                            |
|                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                    | Date of issue<br><b>1980-04-14</b>            |                            |
|                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                    | CODEN:<br><b>LUMEDW/(MESL-1004)/1-85/1980</b> |                            |
| Author(s)<br><b>Kaj Sundqvist</b>                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                  | Sponsoring organisation                       |                            |
| Title and subtitle <b>BLOOD FLOW IN EXPERIMENTAL TUMORS</b><br>With special reference to the effects of vasoactive drugs.<br>A study in the rat.                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                   |                                               |                            |
| Abstract<br>A method for simultaneous determination of cardiac output and regional blood flow in the anesthetized rat is presented, using an intracardiac injection of microspheres labelled with radioactive isotopes while simultaneously a reference sample was drawn from a peripheral artery. The technique was relatively simple to handle in a small animal like the rat and gave good reproducibility. Blood flow in three experimental tumors was studied regarding relation to size, inoculation sites and tumor type. Blood flow was shown to be heterogenic with a greater blood flow in the periphery of the tumor compared to its center. Tumor blood flow was also found to be inversely related to tumor size. This finding was prevalent in liver as well as in subcutaneous tumors. Cardiovascular response and blood flow in normal tissue and tumors were studied after infusion of adrenaline, noradrenaline, glucagon, histamine and vasopressin. Noradrenaline and vasopressin caused a significant decrease in cardiac output and a marked vasoconstriction in normal as well as in tumor tissues. Relative hepatic tumor blood flow, determined as the ratio between tumor and liver blood flow showed a significant increase after noradrenaline infusion and a decrease after infusion of vasopressin. Glucagon, which increased splanchnic blood flow, histamine and adrenaline showed no significant changes in the tumor to liver ratio. In subcutaneous tumors the tumor to muscle or tumor to cutaneous blood flow ratio showed a decrease after infusion of noradrenaline but no change was noted after infusion of any of the other drugs. An absence of adrenergic nerves adjacent to the vessels was observed in hepatic tumors, as determined by fluorescence microscopy, this was further verified by quantitative determination of noradrenaline in tumor and liver tissue. In conclusion it was shown that tumor blood flow was inversely related to tumor size. Blood flow in experimental tumors is heterogeneous with a superior blood flow in the periphery versus the center of the tumor. Of five tested vasoactive drugs, only noradrenaline infusion induced an increase of relative blood flow in hepatic tumors. |                                               |                            |
| Key words<br>Microspheres, cardiac output, regional blood flow, reference sample, hepatic tumors, subcutaneous tumors, tumor blood flow, tumor size, vasoactive drugs, catecholamines, adrenergic nerves.                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                          |                                               |                            |
| Classification system and/or index terms (if any)                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                  |                                               |                            |
| Supplementary bibliographical information                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                          |                                               | Language<br><b>English</b> |
| ISSN and key title                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                 |                                               | ISBN                       |
| Recipient's notes                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                  | Number of pages<br><b>85</b>                  | Price                      |
|                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                    | Security classification                       |                            |

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Date **April 14 1980**

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The thesis is based on the following papers:

- I. Lo Hafström, B Persson & K Sundqvist. MEASUREMENTS OF CARDIAC OUTPUT AND ORGAN BLOOD FLOW IN RATS USING  $^{99}\text{Tc}^{\text{m}}$ -LABELLED MICROSPHERES. Acta Physiol. Scand. 106:123-128, 1979.
- II. K Sundqvist, Lo Hafström & B Persson. MEASUREMENTS OF TOTAL AND REGIONAL TUMOR BLOOD FLOW AND ORGAN BLOOD FLOW USING  $^{99}\text{Tc}^{\text{m}}$ -LABELLED MICROSPHERES. Eur. Surg. Res. 10:433-443, 1978.
- III. Lo Hafström, A Nobin, B Persson & K Sundqvist. EFFECTS OF CATECHOLAMINES ON CARDIOVASCULAR RESPONSE AND BLOOD FLOW DISTRIBUTION TO NORMAL TISSUE AND EXPERIMENTAL LIVER TUMORS. Cancer Res. 40:481-485, 1980.
- IV. Lo Hafström, B Persson & K Sundqvist. THE BLOOD FLOW IN EXPERIMENTAL LIVER TUMORS - THE EFFECT OF VASOACTIVE AGENTS: Preprint Acta Chir. Scand. 146: 1980.
- V. Lo Hafström, B Persson & K Sundqvist. INFLUENCE OF VASOACTIVE DRUGS ON BLOOD FLOW IN SUBCUTANEOUS TUMORS - AN EXPERIMENTAL STUDY IN - RATS. Accepted for publication in J. Surg. Onc.

In the text these papers are referred to by their Roman numerals.



## INTRODUCTION

Treatment of both primary and secondary liver cancer is a surgical and oncological problem of considerable dimensions. Primary carcinoma of the liver is by no means a great quantitative problem in Sweden, where the incidence, although slightly increasing, is around 400 new cases per year (Cancer Incidence in Sweden 1973). Secondary liver cancer, on the other hand, is a very common entity with roughly 3 000 - 4 000 new cases annually. Cancer of the colon and rectum with a number of 3 600 cases per year is by far the most common primary source of metastatic deposits in the liver. Twenty - 25 % of patients with colorectal cancer show synchronous liver metastases at surgery. Survival of untreated cases of primary liver cancer is 1-3 months after diagnosis. Metastatic liver cancer from colon and rectum has about a 6 month median survival (Jaffe et al 1968, Baden & Anderson 1975, Bengmark & Hafström 1979). Despite diagnostic and therapeutic progress, the survival rate for these patients has not increased significantly over the years. Several modalities of treatment have been tried and many of these techniques are based on what is known about the circulation in malignant tumors. Hepatic artery ligation, complete dearterialization of the liver, infusions of cytostatics through the hepatic artery or portal vein have all been tried (Nilsson & Zettergren 1967, Ansfield et al 1975, Almersjö et al 1976, McDermott et al 1978). These techniques have in some reports been claimed to increase survival and also to produce some dramatic remissions (Watkins et al 1970, Cady & Oberfield 1974, Ansfield et al 1975, Almersjö et al 1976, Sundqvist et al 1978). Further knowledge of the vascularity and circulation of hepatic tumors might perhaps give us new means of bringing relief to patients with this disease. It is also of importance to know whether the hepatic blood flow can be manipulated in some way in order to improve the effectiveness of the treatment.

### Different techniques for blood flow measurements.

Several techniques have been used for the study of blood flow distribution in the body. From the initial works by Stewart (1897) using the dilution technique and further elaborations by Hamilton et al (1932) the ground was laid for further research in this field. In 1948 Kety and Smith (1948) provided more information using nitrous oxide in the dilution principle. Sapirstein reported on a technique based on tissue uptake of diffusible ions like  $^{42}\text{K}$ ,  $^{86}\text{Rb}$  and  $^{131}\text{-Iodoantipyrine}$  (Sapirstein 1958). This technique has been used by several other authors also for regional blood flow measurements (Mendell et al 1971, Kjartansson 1976). "The Sapirstein method" gives the regional blood flow in relation to cardiac output, which although it can be established by the same method, is not possible to perform in the same rat (Sapirstein 1958).

Clearance techniques using radioactive inert gases have also been widely used in measurements of blood flow, usually  $^{133}\text{Xe}$  and  $^{85}\text{Kr}$  (Lassen et al 1961, Gump et al 1968, Darle 1970). This technique which is based on intraarterial injection of the isotope has the advantage that cardiac output does not have to be measured, and repeated determinations can be performed in the same animal without sacrificing it. Regional blood flow



in small areas of an organ are, however, difficult to measure with this technique. The  $^{133}\text{Xe}$  method can also be applied by local tissue injection with registration of the clearance curve. This makes it possible to measure the blood flow in restricted parts of an organ. It should be noted that the local injection technique might induce small areas of tissue damage which might influence the results and give large variations in results (Kjartansson 1976). Another possible way is to make the animal breathe a certain amount of  $^{133}\text{Xe}$  and study the wash-out curve over the area of interest after equilibration in the body. This technique is mostly used in cerebral blood flow studies (Eklöf et al 1973). The problem of determining the partition coefficient between the tissue and blood must, however, be recognized (Andersen & Ladefoged 1967, Song et al 1972).

During the last two decades there has been a development of the indicator technique using radioactively labelled microspheres. This technique offers certain practical and theoretical advantages compared with other types of blood flow measurements. Initially it was used to demonstrate arterio-venous shunting and most spheres used were made of glass. A further development of this technique was made when carbonized as well as plastic microspheres were manufactured (Wagner et al 1969). These microspheres could be labelled with different radionuclides. A further development in this field was the introduction of polystyrene (Wagner et al 1969), and dextran spheres (Svedman 1977). The microspheres can be manufactured in different sizes from 2 to more than 100  $\mu\text{m}$ . In small laboratory animals like the rat 15  $\mu\text{m}$  is probably the optimal size to obtain reproducible data without disturbing general circulation. Streaming characteristics of microspheres are also more uniform when this size is used (Phibbs & Dong 1970, Rutili & Arfors 1976). Since the introduction of the microsphere technique was made by Rudolph & Heymann in 1967, it has gained increasing interest. Numerous publications have been produced dealing with the theoretical background and the advantages and disadvantages of the method (Buckberg et al 1971, Archie et al 1973, Mendell et al 1971, Sasaki et al 1971). It appears from the literature that labelled microspheres are suitable for blood flow measurements in small experimental animals.

#### Previous tumor circulation studies

In 1904 Ribbert postulated that tumors have a superior vascular supply compared with normal tissue (Ribbert 1904). This has been accepted for half a decade without really having been proved. In 1923 Segall stated that hepatic tumors were mainly supplied by the hepatic artery (Segall 1923). His study was performed on human autopsy livers. The microvascular architecture of both experimental and human liver tumors has been studied by several authors. Most of these verified the previous findings of a predominantly arterial supply to liver tumors (Bierman et al 1951, Breedis & Young 1954, Honjo et al 1965, Nilsson & Zettergren 1967, Ackerman et al 1969, Lien & Ackerman 1970). This has led to the suggestion that these hepatic tumors could be treated by ligation of the hepatic artery (Markowitz 1952) and this was also carried out in rabbits with experimental liver tumors by Breedis & Young (1954) and later by Nilsson and Zettergren (1967) and others.



The first study of actual blood flow was performed by Gullino and Grantham (1961). They used an ingenious method of cannulating the vein draining a paraffin enveloped tumor grown in the kidney or ovary where the blood supply had been isolated to a single vascular stalk. This method is however artificial and only applicable when the tumor blood is exclusively drained by one vein. Cataland and co-workers (1962) reported on a series of transplantable mouse tumors where blood flow was measured by Sapirstein's method for tissue uptake of diffusible ions. In this study they used the tissue uptake of  $^{86}\text{Rb}$ . Since then several other methods have been used for the purpose of determining blood flow in tumors. Thus Gump and White (1968) used  $^{85}\text{Kr}$  inhalation for determining the wash-out curves and the blood flow in V2-carcinoma implanted in the hind limb of rabbits. The  $^{131}\text{I}$ -antipyrine method was used to determine total blood flow in hamster tumors. It was shown that blood flow decreased with increasing size of the tumor (Rogers 1967). The  $^{86}\text{Rb}$  method as well as  $^{131}\text{I}$ -antipyrine and the  $^{133}\text{Xe}$  method has also been widely used by several authors (Gullino & Grantham 1961, Song et al 1972, Oikawa et al 1975, Kjartansson et al 1976).

In a number of papers Ackerman and co-workers studied the blood supply of experimental metastases in the rat liver (Ackerman et al 1969, Lien & Ackerman 1970, Ackerman et al 1972, Ackerman 1974), using injection of  $^{131}\text{I}$  labelled RISA and also injection of  $^{90}\text{Y}$  labelled microspheres. An arterial supply was noticed in tumors of the liver which became more predominant with an increasing size of the tumor. Their technique was chosen in order to differentiate between portal and hepatic artery flow. They verified their results by perfusion of the tumors via the portal vein or hepatic artery with silicon rubber solution.

In conclusion, substantial evidence does indicate that experimental liver tumors as well as human liver tumors seem to be nourished mainly by the hepatic artery at least when the size of the tumor increases. In rat liver tumors this appears to be a fact when the tumor weight is more than 30 mg. It seems therefore logical to study the arterial blood flow of hepatic tumors.

#### Theoretical background for the microsphere method

The microsphere method for blood flow studies as first described by Rudolph and Heymann (1967) is based on the fact that microspheres are trapped in arterioles and capillaries during the first transit of the blood through the tissues. This technique is based on the tissue distribution of microspheres in relation to the cardiac output which in itself is not determined. The method has been widely used for blood flow measurements in big animals like the cat, rabbit, sheep, goat, pig and dog and to some extent in small animals like the rat and hamster (Rudolph & Heymann 1967, Neutze et al 1968, Kaihara et al 1968, Domenech et al 1969, Sasaki & Wagner 1971, Archie et al 1973). By collecting a peripheral arterial blood sample drawn at constant-speed during the injection of microspheres, cardiac output can be obtained simultaneously with the absolute values of tissue blood flow. This has been done in rather big animals (Hoffbrand et al 1969, Buckberg et al 1971, Archie et al 1973,



Bartrum et al 1974) and only recently has this technique been used in small animals like the rat (Malik et al 1976, Bonaccorsi et al 1978). The technique which is called the reference organ technique has the advantage that the simultaneous determination of cardiac output and blood flow in various organs and parts of organs can be obtained in the same animal.

In paper I the theory behind the microsphere technique is discussed. Briefly the theory is that if the particles are trapped in a given tissue during the first passage the following equation can be devised:

$$q = f \int_0^{\infty} C(t) dt$$

q represents the particles trapped in the organ, f is the blood flow to the organ, and C(t) the concentration of particles in the blood at time t. The Stewart and Hamilton formula describes the cardiac output according to the following equation:

$$Q = F \int_0^{\infty} C(t) dt$$

where Q is the amount of indicator injected and F is the cardiac output. Combining the first two equations gives the following relation:

$$f/F = q/Q$$

If a reference blood sample is collected from a peripheral artery at a known constant withdrawal rate this can be regarded as an artificial organ with a known blood flow. The activity of the indicator in the blood sample which is drawn can be measured and thus the relation is obtained: cardiac output  $F = Q \cdot f_r/q_r$  where  $f_r$  is the withdrawal rate of the reference sample having the activity  $q_r$ . Accordingly, the blood flow of any tissue can be estimated in the same way:  $f_i = q_i \cdot f_r/q_r$  where  $f_i$  is the blood flow of the tissue with the activity  $q_i$ .

The reference organ in the present study was a withdrawal pump of Harvard type drawing the reference sample at a constant rate of  $0.5 \pm 0.005$  ml/min.

The number of spheres injected into the rat is of paramount significance for the results. The specific activity of the injected spheres on the other hand can be chosen within quite wide limits. If count rates of the samples are low the counting accuracy can be improved by longer counting times, by increasing sample weights or by increasing the number of injected spheres. This has been thoroughly investigated by Buckberg et al (1971) and the variability in distribution of spheres to any tissue approximates the Poisson distribution where  $\bar{x}$  is the mean number of spheres reaching the tissue or reference sample and  $\sqrt{\bar{x}}$  is the standard deviation. A confidence interval can be established for the number of spheres that is needed for a given precision at a certain confidence level, e.g., with a confidence level of 95 % and a precision of 10 % the equation  $1.96 \cdot \sqrt{\bar{x}} = 0.1 \cdot \bar{x}$  is obtained; and thus  $\bar{x} = 384$ . That means that in each sample, reference or tissue, a minimum of about 400 spheres is needed for a precision of 10 % at a 95 % confidence level. Similarly the total

number of spheres required for reaching this goal in the animal can be established from the formula:

$$N = F \cdot 400 / f_i \cdot m_i$$

where F is the cardiac output,  $f_i$  is the flow of the sample and  $m_i$  is the sample weight. This means that a great number of spheres has to be injected in small samples with expected low blood flow. In our study we chose to inject 800 000 - 1 000 000 spheres to obtain this precision.



## PURPOSE AND AIM OF THE STUDY

Of the different techniques for the regional blood flow measurements especially in tumors, the microsphere technique using the reference sample method seems most the attractive one, due to a number of factors. The microsphere technique allows several measurements in the same animal which thus acts as its own control in the statistical analysis. This cannot be obtained for example by the  $^{86}\text{Rb}$  method. Although the  $^{133}\text{Xe}$  technique can be used several times, it cannot be used as effectively for regional flow studies as the microsphere technique particularly as it does not allow simultaneous measurements in different areas within an organ or in different organs (vide supra).

Cardiac output determination is of importance in all blood flow studies particularly if manipulation of circulation is going to be performed. This can be done by means of the technique using labelled microspheres and a simultaneously drawn reference sample. Cardiac output determinations cannot be made by using other known methods if a simultaneous regional blood flow determination is required in the same animal. The major disadvantage of the microsphere method for blood flow measurements is that determinations reflect only an instant of the blood circulation. This disadvantage is also found with most other techniques except for the  $^{133}\text{Xe}$  method. The microsphere technique probably gives fewer problems with shunting and recirculation than do other techniques.

As no previous studies regarding tumor blood flow report the use of the microsphere technique with the reference sample method, and as this method seemed to offer certain advantages it was important to establish a blood flow method using  $^{99}\text{Tc}^{\text{m}}$  labelled microspheres for studying regional blood flow and cardiac output in the anesthetized rat.

As experimental as well as human tumors in the liver are predominantly nourished from the hepatic artery and related to increasing tumor size, it seems logical to use the microsphere technique for blood flow measurements in experimental tumors. The aim was to study blood flow in three different experimental tumors in the rat with special emphasis on regional blood flow in the tumor and blood flow in relation to tumor size.

A subcutaneous model for studying therapeutic responses is warranted as this location makes it possible to measure tumor size easily. This model might also be used for the study of distant metastatic spread of the tumor. It is of importance to know about the blood flow of these tumors and the effect that therapy for example cytostatics or X-rays might induce on the flow. The aim was also to study blood flow in different subcutaneous tumors and the relation between tumor size and blood flow.

It has been assumed that vasoactive drugs might influence tumor blood flow and thus be of benefit in the treatment of these tumors by for example cytostatic infusions. Thereby one might obtain increased concentration of the antitumoral agent within the tumor with less toxic side effects. The aim was therefore to study the cardiovascular response to catecholamine infusion with special emphasis on the relative blood flow

in experimental liver and subcutaneous tumors.

As adrenergic nerves in the liver tumors and surrounding liver parenchyma might influence the response to catecholamine infusion, it was of importance to study the adrenergic innervation of experimental liver tumors and their catecholamine content by established methods.

Except for catecholamines, other vasoactive drugs might influence tumor blood flow and thus be used as an adjuvant in cytostatic treatment of tumors. The aim was thus to investigate the cardiovascular response to and the relative tumor blood flow after infusion of histamine, glucagon and vasopressin in liver and subcutaneous tumors.



## MATERIAL AND METHODS

### Animals

Inbred rats of Lister and Wistar strains were studied in all the experiments. The Lister rats were obtained from the University of Gothenburg and originated from the Chester Beatty Research Institute, London. The Wistar rats were obtained from the Wallenberg Research Laboratory, Lund, Sweden. All the rats were fed ad libitum on rat pellets and had free access to water. They were kept in room temperature of about 23°C. Animals of both sexes were used for the experiments and the rat weight was  $223 \pm 4$  g.

### Tumors

In the initial part of the work (paper II) three tumors were studied. One was a benzenpyrene induced sarcoma (BP) and one a hepatoma (Hep-H). Both these were inoculated in Lister rats. The third tumor was a N-methyl-N' Nitrosoguanidine induced adenocarcinoma (NG-W), which was inoculated in Wistar rats. The BP and Hep-H tumors were obtained from the Gothenburg University and the NG-W from the Wallenberg Research Laboratory in Lund. The tumors were propagated by intrarenal transplantation in the appropriate strain of animals. After homogenating the tumor using a trypsin transfer method, 500 000 cells were inoculated into the dorsal part of the rats subcutaneously, and at laparotomy intrahepatically in the left liver lobe. Lymph node metastases developed regionally from all three tumors if left long enough, but spontaneous lung metastases or distant metastases did not occur. Tumor take in the liver was close to 100 % and subcutaneously about 80 %. Tumor growth of these inoculated tumors enabled us to make the blood flow measurements in tumors ranging from 3 - 20 mm. These sizes were obtained after 10 - 20 days depending on tumor type. Because of the aim of the study the tumors in paper II had a wide range of sizes. In paper III, IV and V the Hep-H and NG-W tumors were kept at more constant size of about 10 mm, with an average weight of about 0.8 g. In smaller tumors, i.e., tumors up to 1-1.5 g, no macroscopical central necrosis could be found. Microscopical necrosis could be found even in tumors weighing 0.5 g. In order to check that the tumor characteristics were constant throughout the experimental period, they were controlled periodically regarding degree of malignancy, tumor take, rate of growth, etc. This was done in order to perform studies in as comparable tumors as possible. The characteristics of tumor growth rate and metastatic potential did not change throughout the transfer generations.

### Microspheres

Insoluble, cross-linked dextran microspheres were used for the blood flow measurements. They were delivered, unlabelled, by Pharmacia Fine Chemicals, Uppsala, Sweden as a dry substance in kits.

After swelling the dry microspheres in Tris/acid buffer the radionuclide was added and the microspheres were labelled. A stable metal-chelate complex was then obtained and the labelling was stable for a very long

time. In the present study  $^{51}\text{Cr}$  and  $^{99}\text{Tc}^{\text{m}}$  were used as radionuclides. The  $^{51}\text{Cr}$  isotope has a half life of 27.8 days and the  $^{99}\text{Tc}^{\text{m}}$  isotope a half life of 6 hours. These two isotopes have peak gamma energies which are easy to separate.  $^{51}\text{Cr}$  has a photo peak at 324 keV and  $^{99}\text{Tc}^{\text{m}}$  at 140 keV.

The nominal size of the microspheres was  $15 \pm 3 \mu\text{m}$  according to the manufacturer, but in the present study they were  $14.9 \pm 2.3 \mu\text{m}$ . There were only slight differences between the batches. Virtually no microspheres were bigger than  $20 \mu\text{m}$  and no smaller than  $10 \mu\text{m}$ . The relative density was  $1.12 \pm 0.02 \text{ g/ml}$  and the sedimentation rate was  $2.0 \text{ mm/min}$ . The density is thus very close to that of red blood cells ( $1.09 \text{ g/ml}$ ) and the rheological properties are rather similar to those of the red blood cells (Svedman 1977). In paper I and II only  $^{99}\text{Tc}^{\text{m}}$  labelled microspheres were used for the development of the technique and for characterization of the tumors. In paper III, IV and V the microspheres were labelled with either  $^{99}\text{Tc}^{\text{m}}$  or  $^{51}\text{Cr}$ .

The labelling stability was checked by leaching in 10 % Ficoll<sup>R</sup> for 24 hours and measured in a Sepharose column. Virtually no leaching was noted in vitro. In vivo the labelling stability was checked by measuring the radioactivity of rat urine and also by taking samples from peripheral vein blood 45 min after injection of the spheres. No detectable radiation above background could be registered in these samples. This indicated that the labelling of the microspheres was sufficiently stable.

### Measurements of radioactivity

The initial activity of the syringes containing the labelled microspheres were measured before injection. After injection the residual activity of the syringes, the stop-cocks, and catheter system was measured and subtracted from the initial activity which then constituted the administered activity. The reference samples and the tissue and organ samples were measured in a well-type scintillation counter (Selectronic) with a  $2 \times 2$  inch  $\text{NaI}^{(11)}$  detector connected to a two-channel pulse-height analyzer. Counting times were preset at 1 000 s or 1 000 000 counts. Simultaneous measurements of standards and background were also performed and corrections for these as well as corrections for the half life of the isotopes were added into the computer program which was specially designed for the experiment. Calculation of cardiac output and tissue blood flow were made. Cardiac output was expressed in  $\text{ml} \cdot \text{min}^{-1}$  per 100 g bodyweight and tissue blood flow in  $\text{ml} \cdot \text{min}^{-1} \cdot \text{g}^{-1}$  wet tissue weight. The tumors were measured in the same way as other tissues but after careful dissection from surrounding tissue. Measurements were made in plastic vials with sample heights below 1.5 cm to avoid geometrical errors.

### Drugs

In the drug infusion series (paper III, IV and V) the control group was infused with physiological saline at a rate of  $0.2 \text{ ml/min}$ . The duration of the infusion before the second measurement was as a rule 4 - 5 min. The adrenaline infused rats were given 4 micrograms/kg bodyweight/min and noradrenaline was administered at a dose of 5 micrograms/kg body-



weight/min. Glucagon (Novo Industries, Denmark) was given in a dose of 4 micrograms/kg bodyweight/min, histamine (Vitrum AB, Sweden) in a dose of 2.0 micrograms/kg bodyweight/min and Postacton<sup>R</sup> (Ferring AB, Sweden), a synthetic vasopressin in a dose of 0.04 units/kg bodyweight/min. Infusion times varied somewhat, but circulatory steady-state was as a rule obtained within 3 - 5 min, after which the second blood flow measurement was made. The drugs in these experiments were chosen because of known vascular and circulatory effects. Dosages were based on preliminary dose response studies in 25 rats where the dose chosen induced a maximum vascular and circulatory response with a minimum of side effects and with no deaths in the series attributed to the drug infusion (not published). Side effects from the infusions were slight but could be noticed in nor-adrenaline, adrenaline and vasopressin infused rats, where peripheral vasoconstriction occurred. In two of the histamine infused rats signs of respiratory discomfort were also noted.

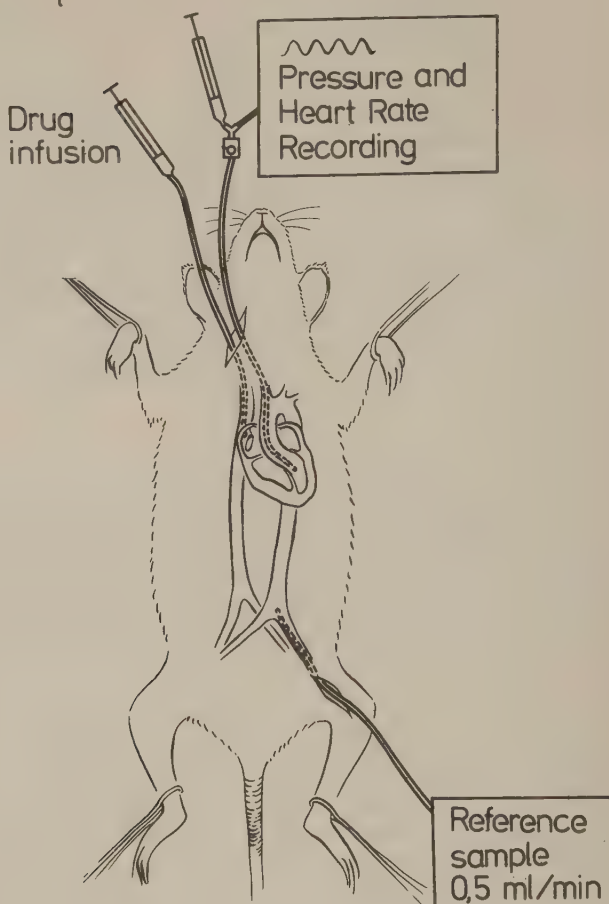
### Preparation of the experiments

The animals were anesthetized with sodium pentobarbital by means of an intraperitoneal injection of 30 mg/kg bodyweight in a 6 mg/ml solution. The rats were placed in a supine position with the paws fixed with needles to the underlying surface. A heating lamp but no heating pad was used. The rectal temperature was maintained at 35°C during the anesthesia. Through an incision in the left groin the external iliac artery and the femoral artery were dissected free and a 0.63 mm (O.D.) polyethylene catheter was introduced into the artery just proximally to the origin of the superficial inferior epigastric vessels. This leaves the leg sufficiently vascularized by collaterals. The catheter was propagated up to the lower part of the abdominal aorta and, after flushing with heparinized NaCl solution, and control of free flow, this was attached to the withdrawal pump. A similar catheter was used for injection of microspheres. This catheter was introduced into the right carotid artery and propagated down into the left ventricle of the heart. The carotid artery was ligated distally. The correct position of the catheter tip was established by means of the pressure curve. The catheter was then connected to a transducer of the Elema-Schönander EMT 34 type and intraventricular pressure and heart rate were monitored throughout the experiment. The experimental set-up that was used in paper I and II was completed in the later papers III, IV and V by an additional catheter introduced into the external jugular vein and progressed down into the superior caval vein. This catheter was used for infusion of vasoactive drugs and in the control series, for infusion of physiological saline, using an infusion pump (Fig 1).

The microspheres labelled with <sup>99</sup>Tc<sup>m</sup> or <sup>51</sup>Cr were injected into the ventricle after vigorous mixing. This injection lasted for about 10 sec, the stop-cock and catheter were then flushed with 0.5 ml of heparinized physiological saline. Just prior to the injection of the spheres the withdrawal pump was started drawing the reference sample at a rate of 0.5 ml/min for about 40 - 60 sec. One minute later the rats were killed by an intracardiac injection of saturated potassium chloride solution. Samples from tissues and whole organs were removed, placed into

Fig 1. Preparation of the experiment. The anesthetized animal with catheters in place and connected to syringes for infusion and registration equipment. The reference sample is drawn at a constant rate of 0.5 ml per min.

15  $\mu$ m microspheres, labelled with  $^{99}\text{Tc}^{\text{m}}$  or  $^{51}\text{Cr}$



plastic counting tubes which were filled with water and counted together with the reference samples in the scintillation counter. Control of catheter positions was carried out in all rats at autopsy and those with catheters wrongly placed were omitted from the series. In series III, IV and V infusion of vasoactive drugs was commenced after the first blood flow measurement. After stabilization of the circulation, measured by blood pressure and heart rate, the second injection with  $^{51}\text{Cr}$  labelled spheres was given and at the same time a reference sample was drawn. In some of the rats  $^{51}\text{Cr}$  was injected first and  $^{99}\text{Tc}^{\text{m}}$  afterwards but no differences were noted in the measurements so the order of isotope injection appears to be of no importance.

#### Adrenergic nerve determinations

In paper III fluorescence microscopical visualization of biogenic mono-



amines was carried out. The technique is based on the Falck-Hillarp method (Falck 1962, Falck & Hillarp et al 1962, Björklund et al 1973). These studies were performed in seven rats. Pieces from liver and tumor tissue were rapidly dissected out and frozen in propane to the temperature of liquid nitrogen. After freeze-drying the specimens were processed for the fluorescence microscopical visualization of adrenergic nerves. Pieces of liver and tumor were also taken for quantitative fluorimetric determination of noreadrenaline, according to the method of Bertler et al (1958, Häggendahl 1963).

#### Preliminary control of the method

The validity of the microsphere method has been widely documented. Even so we had a small series consisting of 5 rats injected with microspheres into the descending aorta. The catheter was positioned at a level of the diaphragm. In this preliminary study the activity of the lungs, which should represent the shunting of spheres in the lower part of the body and the abdominal viscera, was less than 0.5 % of the administered activity, thus indicating a very low degree of shunting or shunts of less than 15  $\mu$ m in size.

No measurements of shunting through the preportal organs were carried out by taking samples from the portal vein, and no check of shunting through the lungs by injection in the venous system for measuring the lodging of spheres in the pulmonary vascular bed. In a few rats, however, a central venous catheter was introduced to the level of the right atrium where a blood sample was drawn simultaneously as the spheres were injected intracardially, and these samples showed virtually no activity indicating that shunting in the peripheral parts of the body was of rather a low degree.

#### Statistical analysis

In the presentation of data the mean and the standard error of mean is given. Test for significance was performed using Student's T-test for paired and unpaired observations. Calculations of correlation coefficients and regression analysis were also performed when needed. Levels of significance \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ .

## RESULTS

In spite of the high number of spheres injected on each occasion no mortality that could be associated with the procedure was registered. Two rats showed signs of excitation during the injection. Fifteen rats had to be rejected during the experimental period because of faults in the catheter positions, technical problems related to injection, infusion or suction of the reference sample. Two rats had to be excluded because of heavy bleeding during the catheterization procedure. Most of these technical problems occurred during the early parts of the experimental series.

Throughout the experiments intraventricular pressure and heart rate were monitored. The average heart rate in anesthetized rats before manipulation with circulation was 350 beats/min, systolic blood pressure was 112 mmHg and cardiac output was 29 ml/min/100 g bodyweight. No significant difference between Wistar and Lister rats was noted, neither were there any differences between tumor-bearing and non-tumor-bearing rats regarding these three parameters.

If all the rats in all series are put together (paper I-V), blood flow of the organs can be summarized as Table I indicates. This shows a rather

|                 | Blood flow<br>$\text{ml} \cdot \text{min}^{-1} \cdot \text{g}^{-1}$ | Cardiac output<br>distribution<br>$\% \cdot \text{g}^{-1}$ |
|-----------------|---------------------------------------------------------------------|------------------------------------------------------------|
| Right kidney    | $3.6 \pm 0.1$                                                       | $6.6 \pm 0.1$                                              |
| Left kidney     | $3.5 \pm 0.1$                                                       | $6.5 \pm 0.1$                                              |
| Myocardium      | $9.0 \pm 0.5$                                                       | $15.1 \pm 0.7$                                             |
| Liver           | $0.9 \pm 0.1$                                                       | $1.1 \pm 0.05$                                             |
| Lungs           | $0.6 \pm 0.03$                                                      | $0.8 \pm 0.004$                                            |
| Spleen          | $1.6 \pm 0.1$                                                       | $1.9 \pm 0.1$                                              |
| Jejunum         | $1.5 \pm 0.1$                                                       | $1.8 \pm 0.1$                                              |
| Bone            | $0.4 \pm 0.03$                                                      | $0.6 \pm 0.04$                                             |
| Pectoral muscle | $0.1 \pm 0.01$                                                      | $0.2 \pm 0.01$                                             |
| Cartilage       | $0.1 \pm 0.01$                                                      | $0.1 \pm 0.01$                                             |
| Skin            | $0.1 \pm 0.003$                                                     | $0.1 \pm 0.004$                                            |

Table I. Blood flow and cardiac output distribution/g in different organs and tissues in anesthetized rats ( $n = 111$ ) Mean  $\pm$  SEM).

high blood flow through the kidneys, myocardium, spleen and the intestine. These measurements were all made before any manipulation with blood

flow was performed. Kidney blood flow was 3.6 ml/min/g and no significant difference between right and left kidney was found. If the right and left kidney blood flow are plotted against each other the regression  $y = 0.89 \cdot X + 0.18$  is obtained. The correlation coefficient is 0.993 ( $p < 0.001$ ). This indicates an almost perfect mixing of spheres. Myocardial blood flow shows rather high values averaging 9.0 ml/min/g. Pulmonary blood flow representing bronchial artery blood flow plus peripheral shunting averaged 0.6 ml/min/g. This is higher than the results obtained in paper I where the pulmonary blood flow was 0.3 ml/min/g ( $p < 0.01$ ). Tumor-bearing rats showed significantly higher pulmonary blood flow compared with non-tumor-bearing rats  $0.7 \pm 0.1$  and  $0.3 \pm 0.05$  ml/min/g respectively ( $p < 0.001$ ). Regarding the splanchnic blood flow both spleen and the proximal part of the small intestine seem to be well nourished with an average blood flow, of 1.6 and 1.5 ml/min/g, respectively. The liver blood flow represented by arterial blood flow plus spheres shunted through preportal organs had an average blood flow of 0.9 ml/min/g. Peripheral tissues such as muscle, cartilage and skin seem to be rather poorly nourished with low blood flow values 0.1 ml/min/g. Bone tissue takes an intermediate position with the blood flow of 0.4 ml/min/g. All these values were based on the whole material before any kind of manipulation of blood flow was performed. Within each subgroup of rats somewhat higher or lower values could be registered, but apart from pulmonary blood flow, as mentioned above, no significant differences between the two strains of rats or differences between tumor- or non-tumor-bearing rats could be observed. There were no differences in arterial blood flow between the liver lobe which carried the tumor and the non-tumor-bearing lobes.

The influence of vasoactive pharmacological infusion on cardiovascular response and blood flow in different organs and in liver tumors was studied in paper III and IV. The cardiovascular response induced by infusion of adrenaline, noradrenaline, glucagon, histamine and vasopressin is summarized in Fig 2 and compared with a control group of rats infused with physiological saline. Adrenaline, noradrenaline and vasopressin increased systolic intraventricular pressure whereas glucagon induced a decrease in blood pressure. Histamine infusion showed no effect on systolic pressure. Heart rate reactions were minimal except in rats infused with vasopressin; these showed a significant decrease in heart rate. Cardiac output decreased significantly in rats infused with noradrenaline and vasopressin. Infusion of adrenaline, glucagon, and histamine induced no significant effects on cardiac output. There were no differences between subgroups of rats, i.e., Wistar and Lister rats, except for the adrenaline infused groups (III).

A summary of the effects on tissue and organ blood flow after infusion of five vasoactive drugs compared with saline infusion is shown in Fig 3 (paper III and IV). A significant decrease in the blood flow of the kidneys, lungs, liver and spleen was noted in the noradrenaline infused rats, whereas adrenaline showed a decrease in pulmonary, intestinal and splenic blood flow. Kidney and liver blood flow were unaffected. Myocardial blood flow was increased by the catecholamines but also by saline infusion. Vasopressin infusion induced a significant decrease in all organs and tissues measured. Glucagon induced an increase in splenic and intestinal blood flow whereas the pulmonary blood flow was decreased. Ar-



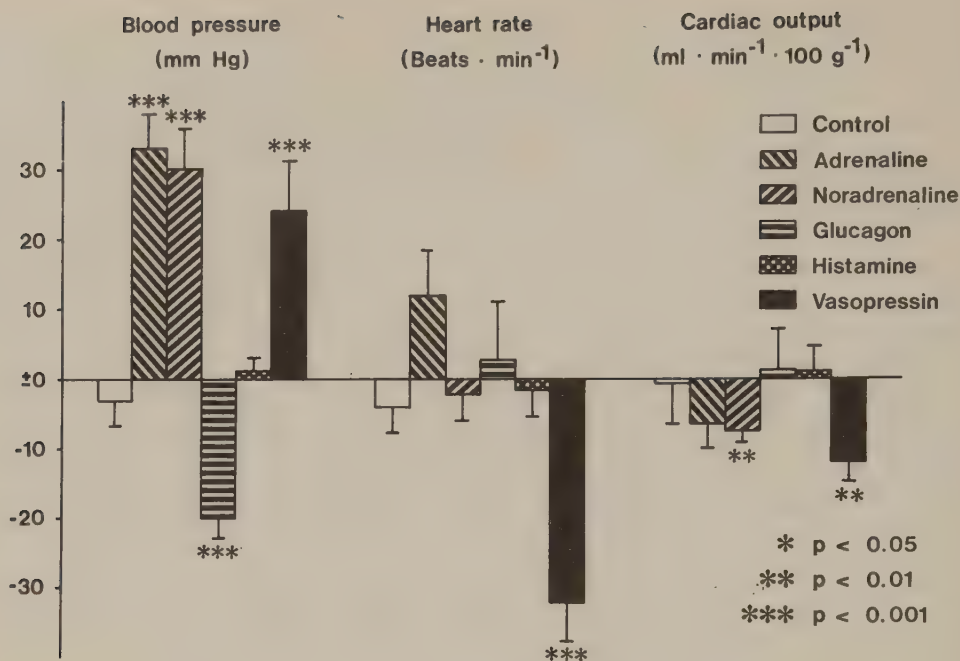


Fig 2. Influence on systolic intraventricular pressure, heart rate and cardiac output of infusion of physiological saline and vasoactive drugs. The bars indicate mean differences  $\pm$  SEM before and after infusion.

terial liver, myocardial and kidney blood flow were not affected. Histamine infusion decreased liver and pulmonary blood flow but left all the other organs unaffected.

Blood flow in different tumors implanted in the liver and subcutaneously was studied with regard to size. In smaller liver tumors total arterial blood flow was studied in 70 tumors with an average weight of 0.77 g. Average blood flow in these tumors was in general lower than in surrounding arterial liver tissue blood flow. Blood flow in these tumors was inversely related to size (Fig 4). The same pattern was noted in the Hep-H and NG-W tumors. Blood flow of the liver tumors could be described as an exponential function;  $y = 0.62 \cdot e^{-0.58 \cdot m}$ .

In subcutaneously implanted tumors of less than 1.5 g, a picture similar to that of hepatic tumors was noted. Total blood flow in these tumors with an average weight of 0.73 g was also inversely related to the weight of the tumor (Fig 5). The exponential function was  $y = 0.54 \cdot e^{-0.67 \cdot m}$ . Subcutaneously implanted tumors showed a 4 - 5 times greater blood flow than that of surrounding tissue, i.e., skin or muscles.

Of the three tested tumors the BP sarcoma showed a somewhat higher blood flow than the two other tumors tested. The same relationship of total

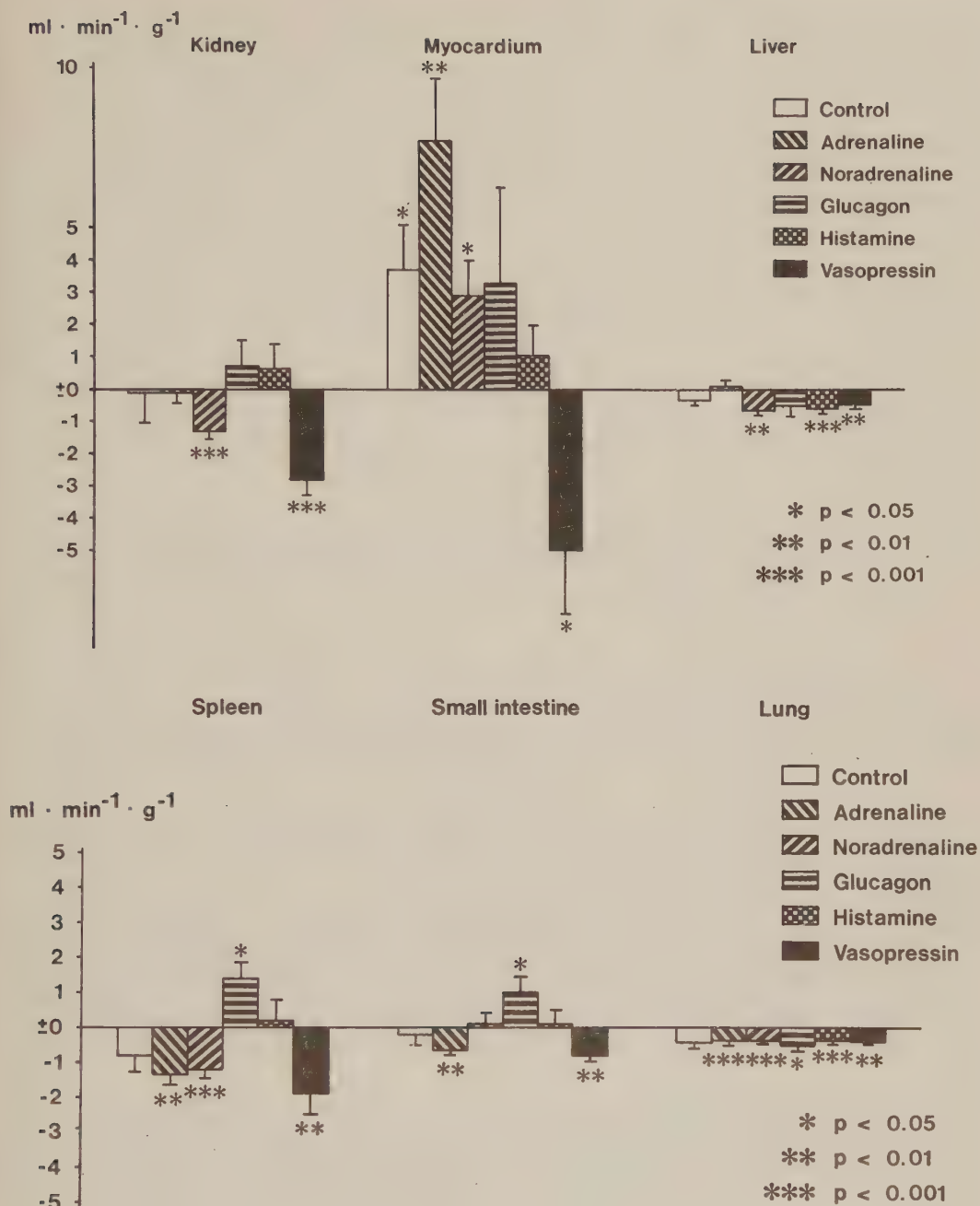


Fig 3. Effects on blood flow in different organs of infusion of vasoactive drugs and as control physiological saline. The bars indicate mean differences  $\pm$  SEM before and after infusion of the drugs.

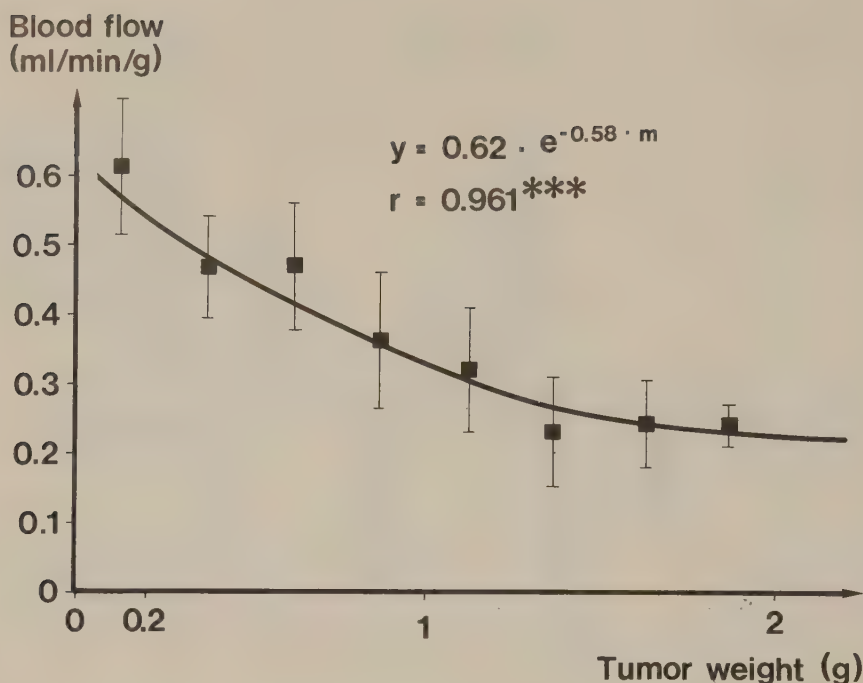


Fig 4. Arterial blood flow in liver tumors as a function of tumor weight. Values are given as mean  $\pm$  SEM in each weight range. The best fit equation is  $y = e^{-0.58 \cdot m}$ .  $m$  = tumor weight.

blood flow in relation to size was prevalent, both in intrahepatic and subcutaneous tumors.

In bigger tumors (> 1.5 g) blood flow in the periphery of the tumors was significantly higher than central blood flow. This was the case in both liver and subcutaneous tumors (paper II). Blood flow in lymph node metastases was also measured but showed no definite trend regarding blood flow as a function of tumor weight. Average blood flow was 0.42 ml/min/g and the average weight was 0.46 g.

Blood flow in liver tumors and subcutaneous tumors was studied after infusion of adrenaline, noradrenaline, glucagon, histamine, and vasopressin (paper III, IV, V). This was compared with a control group infused with physiological saline. In liver tumors, infusion of the different drugs induced blood flow changes which were similar to those of the surrounding liver tissue. The ratio between tumor and surrounding liver tissue blood flow as well as between subcutaneous tumor and skin and muscle blood flow was calculated and these results showed that only after noradrenaline infusion and vasopressin infusion were there any significant changes in the liver tumors. Thus noradrenaline induced a significantly increased tumor to liver blood flow ratio indicating a preferential blood



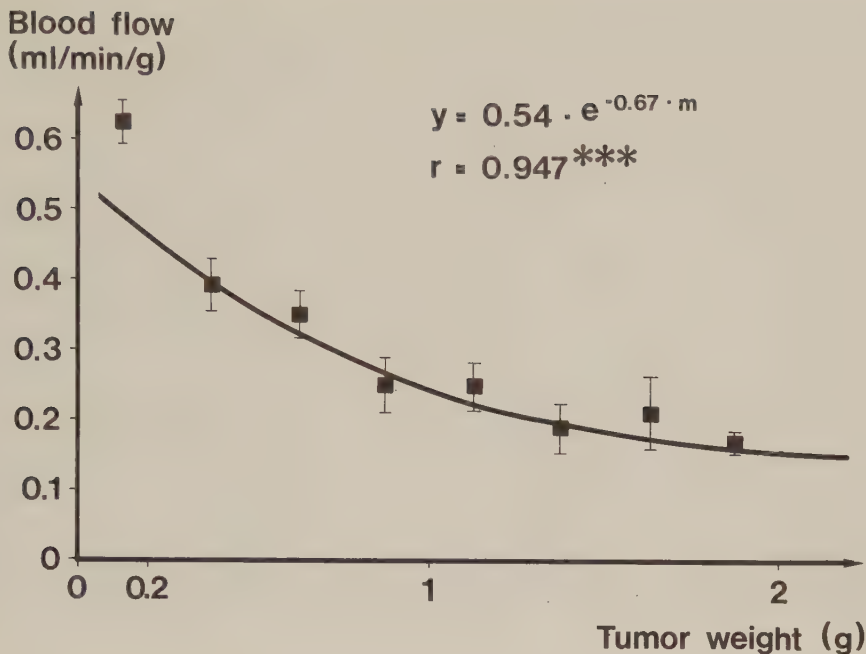


Fig 5. Blood flow in subcutaneous tumors as a function of tumor weight. Values are given as mean  $\pm$  SEM in each weight range of the tumor. The best fit equation is  $y = 0.54 \cdot e^{-0.67 \cdot m}$ ,  $m$  = tumor weight.

flow to the tumor. Vasopressin, on the other hand, decreased the tumor blood flow more than the surrounding liver blood flow as calculated by the tumor to liver blood flow ratio. In subcutaneous tumors a similar picture was noted, i.e., a decrease of blood flow in surrounding tissues as well as in the tumors when vasoconstrictors were infused. Tumor to surrounding muscle and surrounding skin blood flow ratios were not affected except in rats infused with adrenaline that showed a decreased tumor to skin blood flow ratio and those infused with noradrenaline that showed a decreased tumor to muscle blood flow ratio. In these studies no differences were noted when analysing the subgroups with hepatoma and NG-W tumors.

In paper III the Falck-Hillarp method for the visualization of biogenic monoamines on the intracellular level was used on liver and tumor in seven rats. The investigation showed that a dense network of noradrenaline-containing nerves was prevalent in connection with the blood vessels in normal liver tissue (Fig 6) but no innervation of noradrenaline-containing nerves could be found adjacent to blood vessels in the tumors. These findings could be verified by chemical determination of the noradrenaline content. Normal rat liver contained 0.15 microgram noradrenaline per gram liver tissue while the noradrenaline content was not detectable in tumor tissue.

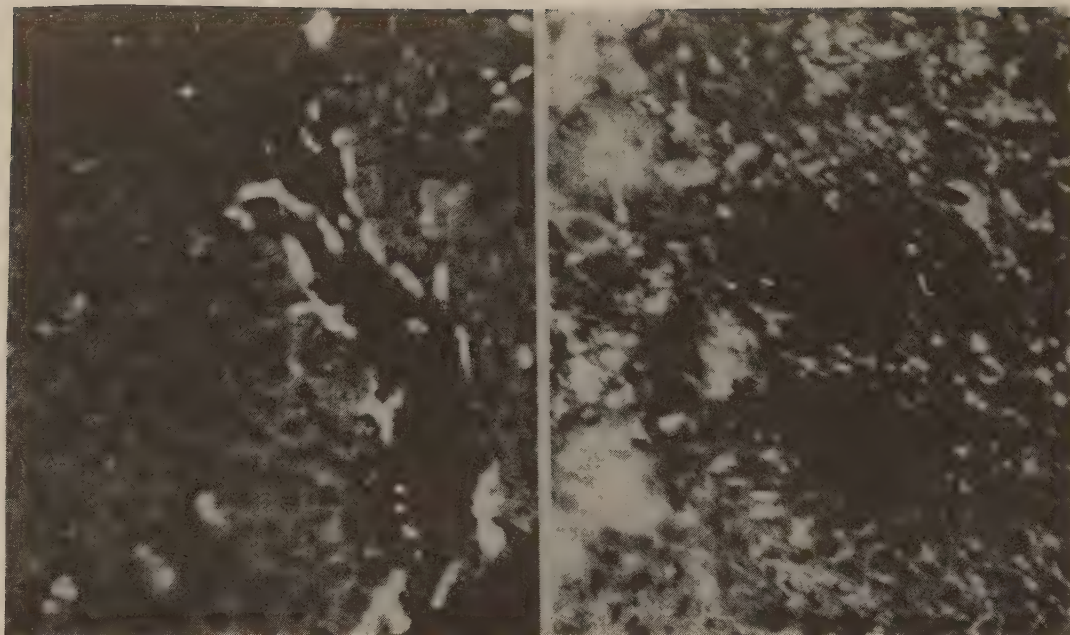


Fig 6. (Left) Fluorescence photomicrograph of normal liver parenchyma in the rat. Fluorescence specific for noradrenaline can be seen adjacent to the vessel wall. (Right) A fluorescence photomicrograph on an adenocarcinoma (NG-W) induced in the rat liver. No fluorescence can be seen adjacent to the two vessels visible in the picture. (x 180)

## DISCUSSION

### The microsphere technique

Blood flow measurements with radioactive tracers have been widely used during the last 3 - 4 decades. These tracers are often bound to different carriers. The methods have been refined and new radioactive isotopes and vehicles for their distribution in the body have been introduced, and the latest design in this field seems to be the use of radioactively labelled microspheres (Bassingthwaite 1976). This technique is now generally accepted as a safe and accurate method for experimental designs (Rudolph and Heymann 1967, Nutze et al 1968, Kaihara et al 1968, Domenech et al 1969, Sasaki and Wagner 1971, Archie et al 1973, Bartrum et al 1974, Malik et al 1976).

The microsphere method for measuring blood flow is based on some basic assumptions:

- 1) the microspheres mix uniformly with the blood before leaving the heart,
- 2) the microspheres are completely extracted from the blood during the first transit of the tissue and do not recirculate,
- 3) the injected microspheres themselves do not significantly affect blood flow.

To test the assumption of uniform mixing of the microspheres with the blood, different techniques have been applied. Thus Shibata and MacLean (1966) demonstrated this uniform distribution of microspheres in the lung following intravenous injection of radioactive microspheres. The technique was based on measurements of multiple small tissue samples taken from the organ. Phibbs and co-workers (1970) also evaluated the mixing of spheres with arterial blood by instant freezing of the artery in which the microspheres were in transit. Thin sections of the arteries were studied, and they found that bigger spheres tended to accumulate in the center of the blood stream and smaller particles along the vessel walls. They concluded that the flow of spheres from a medium sized artery into a smaller branch is similar to the blood flow despite the rather high specific gravity of the spheres used in their study (1.1-1.6). They found no sedimentation of the microspheres. Smaller spheres around 10  $\mu\text{m}$  behaved more like red cells of the blood. It is thus important to keep a very narrow distribution of microsphere size in order to minimize non-random distribution of spheres in the blood stream. It is also important to use microspheres that do not differ too much in density from red blood cells. The spheres used in this study seemed to be ideal from this point of view which is in agreement with the conclusion drawn by Svedman (1977).

An equal distribution of spheres in paired organs can also be used as an index of uniform mixing (Bartrum et al 1974, Warren and Ledingham 1974). In the present study the relation between right and left kidney blood flow showed correlation coefficients close to 1.0 indicating an equal distribution and a uniform mixing.



That microspheres are extracted and impacted in the capillary bed during the first transit of the tissues is an important prerequisite for blood flow measurements using the microsphere method. This has been studied by several authors using spheres of different sizes and in different species. Warren and Ledingham (1974) studied 15, 25 and 50  $\mu\text{m}$  spheres in the rabbit and found shunting to the lungs primarily through the ears and hindlimbs. Shunting was greatest for the smallest microspheres but could be decreased by sodium pentobarbital anesthesia. Archie et al (1973) and Buckberg et al (1971) noted less than 2 % shunting of spheres in dogs and lambs. A total shunting through peripheral tissues in the rat was about 2 % of cardiac output distribution (Svedman 1977). The present study (paper I) as well as methodological studies (not published) have indicated a very low degree of shunting in rats under pentobarbital anesthesia. The total cardiac output distribution per gram tissue in the lungs was 0.4 % and this corresponds to about 1 % of cardiac output to the whole organ which indicates a low degree of shunting. A similar figure 0.93 % was reported by Bartrum in rabbits (1974). The microsphere technique is not aimed to study bronchial artery blood flow when as much as 1 - 2 % of the spheres in the rat might be shunted through peripheral tissues to the lungs (Svedman 1977).

Microspheres themselves do not seem to affect circulation if an appropriate number of spheres with an appropriate size is injected. Thus Kaihara et al (1968) noticed no effect of repeated injections of spheres using microspheres labelled with different nuclides. In dog experiments these authors also studied the distribution of microspheres of different sizes from 15  $\mu\text{m}$  up to 190  $\mu\text{m}$ . In a similar study in rats by Sasaki et al (1971) a similar effect was obtained after repeated injections except for the uptake of labelled spheres in the kidney which dropped after the second injection. They used, however, spheres that were bigger than in the present study (50  $\mu\text{m}$ ), and the number of spheres was also substantially lower. Svedman (1977) used 500 000 - 700 000 spheres without noticing serious side-effects except for agitation and hyperventilation in three rats. Lindell (1977) used 450 000 spheres without side-effects in Wistar rats. Forsberg (1978) using 900 000 tracer sephadex 15  $\mu\text{m}$  spheres noticed no side-effects from the injection in Sprague-Dawley rats under pentobarbital anesthesia. In the present study 800 000 - 1 000 000 spheres were injected on each occasion without serious side-effects. It is concluded that injections of less than 1 000 000 15  $\mu\text{m}$  spheres are fairly safe in small animals and they can, without risk, be repeated several times.

A high number of spheres needs to be injected on each occasion in order to keep statistical errors within reasonable limits. It has been stated by Buckberg et al (1971) that a minimum number of 400 spheres per sample is needed. In order to minimize the effects of spheres it is also wise to use particles that have a specific weight close to that of blood cells. Concerning the Tracer Sephadex spheres used in this work these prerequisites seem adequate.

Blood flow measurements using microspheres in small animals like the rat are based on catheterization of the left ventricle of the heart. This

catheter is introduced via the right carotid artery which means that the right carotid must be ligated distally. The catheterization of the heart per se does not seem to interfere with the cardiac function as no disturbances in central hemodynamic parameters were observed. Only occasionally have arrhythmias been noticed. In spite of the ligation of the right carotid artery, the same rate of blood flow is registered in each of the cerebral hemispheres (Malik et al 1976, Svedman 1977). This indicates that one carotid is enough for the nutrition of the brain in rats. Experiments with ligation of both carotid arteries have also been performed and this technique seems to diminish blood flow through the brain (Malik et al 1976, Svedman 1977), but no alterations in blood flow through other organs than the brain were noted. The only circulatory parameter that was disturbed by bilateral ligation of the carotids was a slight increase in blood pressure; heart rate was not affected (Malik et al 1976).

The method for blood flow measurements using microspheres is based on an intracardiac catheter. This catheter has to be sufficiently small so that it does not interfere with the blood stream through the aortic cusps. In the present study, as well as in several other published works, a small polyethylene catheter with an outer diameter of 0.63 mm has been used. This corresponds to the PE-10 catheter used by Sasaki et al (1971) and others. The position of the catheter was initially checked by the pressure curve but later in the study when the techniques were standardized it could be established by looking at the pulse wave in the catheter. The effect of the catheter on blood flow measurements has been studied by Kaihara et al (1968) and rather high counts for the myocardium were obtained. This might be due to a direct lodging of spheres in the venae minimae and by deposition of spheres in the ventricle wall and in the papillary muscles, or by inadequate mixing at the orifices of the coronary arteries. Because of that it was stated that myocardial blood flow measurements are better done with the catheter in the left auricle (Kaihara et al 1968, Hoffbrand & Forsythe 1969).

### Environmental effects

Blood flow measurements can be affected by the anesthetic agent used during the experiment. A redistribution of blood in the rat by pentobarbital anesthesia was found by Sasaki and Wagner (1971) studying blood flow distribution in rats with or without anesthesia. They found that an increased fraction of cardiac output was delivered to the lungs and the splanchnic organs and a decreased blood flow fraction to the brain during anesthesia. Liver and kidney blood flow did not seem to be affected. Similar results have also been obtained in rabbits by Ardahl et al (1973). They found a redistribution caused by pentobarbital anesthesia from skeleton muscles to kidneys and the intestinal tract. Zannelli et al (1975) found the same in mice and also an increased fraction to mouse tumors by a factor of 1.3-2.0. Warren and Ledingham (1975) on the other hand, found that pentobarbital anesthesia induced a fall of about 26 % in total renal blood flow measured by the microsphere technique in rabbits. It is thus of importance to recognize these changes induced by anesthesia when analysing the results of blood flow measurements reported by different authors. The effects on blood flow induced by different

drugs might also be influenced by the anesthesia. It is obvious that the results must be viewed with this in mind. In paper III there was an unexpected decrease in cardiac output in one of the rat strains, noticed after adrenaline infusion, which might be explained by a concomitant action of the pentobarbital anesthesia.

The body temperature is another factor that can influence central hemodynamics and the blood flow to different tissues. This has been studied under varying circumstances when body temperature of experimental animals drops considerably or rises to values around 40°C. Bonaccorsi et al (1978) found that a sudden increase of temperature induced a significant increase in total cardiac output and a selected increase of blood flow in tissues that are involved in the heat regulation in the rat. Lowering of the environmental temperature to 4°C on the other hand induced few changes in blood flow distribution, and cardiac output was not affected. The experiments in the present study were performed with an effort to keep the environmental temperature stable in order not to lower the body temperature. According to Bonaccorsi (1978) and Aardahl (1973), a drop in the body temperature of about 2°C as observed in the present study is of no importance regarding cardiac output and regional blood flow.

Measurements of cardiac output in rats have previously been chiefly done with dye dilution techniques. The reference organ technique using labelled microspheres makes it possible to measure cardiac output simultaneously with tissue or organ blood flow measurements. This can be performed repeatedly by using spheres labelled with different radionuclides with varying  $\gamma$ -energies. Measurements can be carried out in anesthetized as well as in non-anesthetized animals. In anesthetized animals cardiac output measured by Sapirstein's method using  $^{86}\text{Rb}$  was 20.5 ml/min/100 g (Sapirstein 1958) and 21 ml/min/100 g was noted by Bullard (1956) using indicator dilution methods. In the present study, cardiac output was 29 ml/min/100 g bodyweight. These rats were lightly anesthetized with pentobarbital. Malik et al (1976) using the same technique registered a cardiac output in non-anesthetized rats of 27.8 ml/min/100 g bodyweight. Norlén et al (1978) noticed a cardiac output of 29 ml/min/100 g bodyweight in rats anesthetized with sodium thiobutabarbital. Forsberg et al (1978) found a cardiac output of 80 ml/min in rats with an average weight of about 300 g. These rats were anesthetized with sodium pentobarbital in a slightly higher dose than in our experiments. There seems to be a good agreement between these and our results indicating that the technique used is reliable. In the present series there were no differences in cardiac output between tumor-bearing and non-tumor-bearing rats, suggesting that shunting was so low that it did not affect general circulation, including cardiac output. A certain degree of shunting in the tumors occurred, however, as can be observed in the tumor-bearing rats where the pulmonary distribution of isotopes was significantly higher than in non-tumor-bearing rats. A low degree of shunting or an absence of shunting was observed by Oikawa et al (1975) in a Walker rat carcinoma implanted in the hind leg using the  $^{133}\text{Xe}$  technique. This is contradictory to some other findings where arterio-venous shunting in human as well as experimental tumors has been observed (Kligerman et al 1961, Göthlin 1979).



The present investigation does not solve the problem of detecting shunts, but most data indicate that shunting of blood through tumors exists but the size of the shunts might vary. In order to make correct interpretation of these data the method for blood flow measurements must be known.

### Tissue blood flow

Blood flow in different tissues and organs measured in the present study show results that are quite similar to others reported in rats under anesthesia (Mendell and Hollenberg 1971, Sasaki and Wagner 1971, Forsberg et al 1978, Norlén et al 1978). There seems to be a somewhat higher blood flow in the intestinal tract and the liver in non-anesthetized rats (Sasaki and Wagner 1971, Malik et al 1976, Svedman 1977). This is probably due to a redistribution of blood from skeletal muscles caused by the anesthetic agent.

In the present study with a catheter placed in the ventricle some errors might be induced regarding myocardial distribution of radioactivity which can explain the high myocardial blood flow obtained. Inadequate mixing at the orifices of the coronary arteries might be one of the causes (Kaihara et al 1968, Hoffbrand and Forsythe 1969). It might also be due to lodging of particles within the venae minimae in the myocardium, or in the papillary muscles. A better mixing of the spheres at the orifices of the coronary arteries might be obtained if the catheter is placed in the left auricle instead. For technical reasons this cannot be performed in the rat but may well be accomplished in bigger animals.

As the activity of the lungs is represented by the bronchial artery flow plus spheres shunted through peripheral existing shunts the pulmonary blood flow is not very suitable for studying with the microsphere method. This degree of shunting has been evaluated by others using 15  $\mu$ m spheres in the rat and seems to be less than 2 % (Buckberg et al 1971, Archie et al 1973, Svedman et al 1977) a figure borne out in the present study. There were, however, some differences between tumor- and non-tumor-bearing rats indicating that additional shunts in the tumors might exist (vide supra).

Liver blood flow determined by the microsphere method represents the arterial blood flow plus a rather limited shunting of spheres through pre-portal organs (Lindell et al 1977). The overall arterial blood flow of the liver was 0.9 ml/min/g. This figure is much higher than that observed in non-anesthetized rats, and can be explained by the anesthetic agent, which causes a redistribution of blood flow to the gastrointestinal tract (Malik et al 1976, Forsberg et al 1978). Total liver blood flow can also be obtained using the microsphere method, but then portal flow must be indirectly determined by adding the preportal organ blood flows (Malik et al 1976).

Cardiovascular response after infusion of catecholamines showed effects which were most prominent in the noradrenaline infused animals. Noradrenaline which is a potent alpha-receptor stimulant causes an increase in peripheral resistance with a concomitant increase in blood pressure and

a tendency to bradycardia by means of a reflex mechanism (Goth 1978). The similar pattern that was noted after adrenaline infusion, although exerting its effect on alpha- and beta-receptors, can be attributed to the rather high dose of adrenaline infused, which tends to abolish the differences between noradrenaline and adrenaline (Goth 1978). The increased myocardial blood flow which was noticed might be explained by the increased heart labor induced by the catecholamines.

The effects of glucagon infusion in the anesthetized rats were most prominent in intestinal and splenic blood flow which decreased significantly. The arterial blood flow of the liver was, however, unchanged. This, in combination with the results on total hepatic blood flow obtained by Ohnhaus (1972) using the  $^{86}\text{Rb}$ -method, indicates that the increased total blood flow of the liver is attributed to an increase of the portal blood flow. Effects of histamine infusion on central hemodynamic parameters and cardiac output were negligible which is expected as the rat is rather resistant to circulatory effects of histamine (Goth 1978), although a decrease in liver and pulmonary blood flow was noted. Vasopressin, the most potent vasoconstrictor, caused an increase in blood pressure with a concomitant reflex bradycardia and decreased cardiac output. Vasopressin, which exerts its effect probably directly on the smooth muscles in the vascular wall, caused a marked decrease in blood flow to the myocardium and the abdominal organs including the arterial flow in the liver. The latter finding is somewhat contrary to that of Eriksson (1971) who found an increase in hepatic arterial flow in the dog.

#### Tumor blood flow

It has previously been shown in injection experiments by Breedis and Young (1954) that the blood supply of neoplasms in the liver is mainly of arterial origin. These studies were performed on VX-2 rabbit carcinoma and on T-241 sarcoma in mice. It was also noticed that the blood supply of the transplanted tumors was of arterial origin whether the inoculation was made via the hepatic artery or via the portal vein or directly into the hepatic parenchyma. Similar results have also been obtained by others (Krishna Murthy 1959, Honjo and Matsumura 1965, Ackerman 1970). The general impression is that the blood supply of liver tumors seems to become more arterial as the tumor increases in size (Ackerman et al 1969, 1974). In small tumors some portal vein supply seems to exist (Honjo and Matsumura 1965, Nilsson and Zettergren 1967, Ackerman 1969). These findings were confirmed by results obtained when the hepatic artery was ligated causing necrosis of the tumors (Nilsson and Zettergren 1967).

The present study was designed to evaluate the arterial circulation of tumors in relation to their size, location and type. The three tumors that were studied; the NG-W adenocarcinoma, the Hep-H hepatoma and the benzopyrene induced sarcoma showed a similar pattern with a decreasing relative tumor blood flow as the size of the tumor increased. This finding was consistent in all the experiments before manipulation of the blood flow, and could be described as an exponential function. This relationship was evident in all tumors studied. There were, however, some differences between the three tumors regarding blood flow. Thus the

benzopyrene sarcoma showed a higher blood flow both in subcutaneously and intrahepatically implanted tumors than both the NG-W and the Hep-H tumors (II). This study dealt with whole tumors of a small to moderate size (0.1-1.5). In bigger tumors regional blood flow within the tumor was investigated. The result was a significantly higher blood flow in the peripheral parts of the tumors than in the central parts where necrosis was prevalent. It was also noted that the arterial blood flow in liver tumors was considerably lower than that of surrounding normal liver tissue. It was also evident that blood flow in all three tumors of the same size but weighing less than 1.5 g had the same blood flow whether they were implanted in the liver or subcutaneously. In bigger tumors there were some differences between intrahepatic and subcutaneous growth sites regarding blood flow in the periphery. Subcutaneously implanted tumors showed a lower flow than the intrahepatic tumors in the periphery. The central parts of both these types of tumor had blood flow of equal magnitude. This difference between hepatic and subcutaneous tumors might be due to the superior vascularization of the liver compared to subcutaneous tissue.

Similar results concerning the relation of size to blood flow were obtained by Cataland et al (1962), using the  $^{86}\text{Rb}$  technique in a mouse carcinoma and a mouse sarcoma, and by Rogers and co-workers (1968) in hamster tumors using a combined method of  $^{131}\text{I}$ -antipyrine injection and of radioactive microspheres and Lissamine green injection. However, in a study of blood flow in a Guérin carcinoma in rats, Takács and co-workers (1975) noticed no difference in blood flow between small and big tumors. The size of the tumors was, however, rather large with an average weight of about 1.21 g and this lies in the upper range of the tumors in the present study. Nevertheless there seems to be some evidence that different tumors may have different types of circulation. The general impression is that most tumors have a blood flow that is rather lower than that of most of the surrounding tissues where they are implanted, especially those that are well nourished, for example the liver (Plengvanit et al 1972, Kjartansson 1976). This in-homogeneous blood flow distribution within tumors has also been noted by O'Brian and Weall (1974), and was also found by Kjartansson (1976) using the  $^{86}\text{Rb}$  and  $^{125}\text{I}$ -antipyrine method in a 20-methyl-cholantrene-induced sarcoma and hepatoma implanted in the hind-leg of rats.

### Morphological aspects

Studies of the morphology of experimental tumors have shown an absence of muscular and nerve tissue adjacent to the tumor vessels (Krylova 1968). It seems, however, that different tumors might have different morphologies. Thus Intaglietta et al (1977) using the Algire chamber technique found that arteriolar vessels incorporated in tumor mass showed no structural changes. Falk (1977) using microangiographic techniques could show that in a primary sarcoma both arteries and veins retained muscular walls until branching became fine in the fibrosarcoma studied, but in two other tumors he found no obvious muscle layer in the intratumoral blood vessels. Mattsson et al (1977) studied in rats the microvascular architecture in a hepatoma and a 20-methyl-cholantrene induced sarcoma by means of micro-



angiographic and histochemical techniques for visualization of catecholamines. They could show that in the border between muscle and tumor the arterioles were innervated by adrenergic nerves but the intratumoral vessels lacked these nerves. The same findings were found in the present study in the hepatoma and the NG-W adenocarcinoma. This observation was further verified by measurements of catecholamine contents in the tumors. These findings, however, do not exclude the presence of adrenergic receptors in the tumor vessels. Wickersham et al (1977) found a marked difference in vasoconstrictor ability between neoplastic and normal mammary tissue. This response was more pronounced with topical than with intravenous administration of vasoactive drugs. It was also more prominent in arterioles than in venules and more pronounced in bigger tumors. Adrenaline and noradrenaline applications showed the same effect. The response of vasoconstrictors given intravenously is probably due to a constriction of the arterioles in the normal innervated pretumoral vessels, while the effect of topical application might be due to the presence of adrenergic receptors in the tumors.

The present study showed a decreased blood flow through the hepatic tumor tissue but an even greater decrease in blood flow of the normal hepatic tissue after noradrenaline infusion which resulted in a decreased tumor to liver ratio. This difference in response between tumor and normal tissue might be explained by the absence of smooth muscles or the absence of adrenergic nerves along the intratumor vessels but does not exclude the existence of alpha-adrenergic receptors in the intratumoral vessels.

#### Vasoactive drugs and tumor blood flow

The effect of catecholamines on blood flow in experimental tumors has been studied by several authors. Rankin et al (1976) noted a 13-fold increase in resistance of the tumor vascular bed after administration of noradrenaline. Cater et al (1962) also found a rapid fall in tumor blood flow after intravenous injection of noradrenaline and adrenaline. This was measured by changes in tumor oxygen tension. Ackerman has performed a series of experiments in a Walker rat carcinoma implanted in the liver. In a study published in 1977 he found that adrenaline, bradykinin and glucagon appeared to open capillary-like vessels in the central part of the tumor while at the same time normal vessels outside the tumor area reacted with contraction or dilation depending on the agent given (Ackerman et al 1977). Mattsson et al (1978) used noradrenaline infusion and found that the distribution of blood flow was reduced significantly both intramuscularly and in tumors implanted in the muscle. This was counteracted by pretreatment of the animals with an alpha-receptor blocker. The effect of the intravenous infusion of noradrenaline might be explained by the direct action on normal tissue in the transplantation area which in Mattsson's case was muscle and in the present study liver and subcutaneous tissue. Mattsson's experiments were repeated by a modification of a pletysmographic technique (Kjartansson 1976) where simultaneous measurements of total blood flow in both hind paws were made under ether anesthesia. Total tumor blood flow was calculated as the difference between blood flow in the normal hind leg and in the tumor-bearing hind leg. This study showed a comparatively small change in the tumor blood flow

whereas a considerable decrease was noted in the normal limb blood flow. In a recently presented paper Mattsson et al (1979) used injections of a mixture of noradrenaline and  $^{133}\text{Xe}$  into the tumor for establishing the local tumor blood flow. By this technique the direct effect on tumor vessels could be studied. This experiment showed that both normal tissue (muscle) and tumor vessels reacted with a constriction indicating that in spite of the lack of adrenergic innervation, tumor vessels might be influenced by vasoactive drugs, possibly by direct action on alpha-adrenergic receptors. The present study of subcutaneous tumors showed, that in hepatic tumors there was a marked decrease in tumor blood flow after administration of catecholamines. The tumor to muscle or tumor to skin ratios were, however, not increased. Thus no improvement of tumor blood flow in relation to surrounding tissue could be noted. This might be explained by the fact that subcutaneous tumors are usually supplied by one or perhaps only a few arterioles (Falk 1977, 1978). This preneoplastic artery has normal smooth muscles in the walls and reacts normally to catecholamines, which might explain the "normal" reaction of blood flow in these tumors.

The effect of glucagon, histamine and vasopressin on subcutaneous tumor blood flow was with the exception of glucagon a decreased blood flow but the tumor to skin or tumor to muscle ratios were, however, not affected. Regarding intrahepatic tumors the effect of glucagon and histamine was minimal or absent but vasopressin decreased the tumor to liver ratio significantly. This effect on tumor vessels seems puzzling as vasopressin probably exerts its effects directly on smooth muscles in vascular walls (Goth 1978). If tumor vessels lack smooth muscles, the supposed effect would be the opposite. Vasopressin is, on the other hand, a potent vasoconstrictor and if the general tumor blood supply is lower than that of normal tissue, then it is possible that the pretumoral vessels which are strongly contracted by the action of vasopressin in this way, might diminish tumor blood flow more than the blood flow to normal tissues.

All the studies presented in this work were performed on animals under pentobarbital anesthesia and the effect of the anesthetic agent must be recognized. It is, however, not likely that tumor circulation would be more affected by anesthesia than for example normal tissue blood supply. On the other hand, anesthesia has a depressing action on the heart and lowers general blood pressure thereby it could affect the tumor circulation selectively (Vaupel 1975, Zannelli and Fowler 1977).

Intravenous infusion of vasoactive agents in rats under pentobarbital anesthesia can induce general effects on central circulation and also regional blood flow as well as effects on tumor blood flow. Most vasoactive drugs in the present study caused constriction of normal as well as tumor vessels which is due to direct action on smooth muscles in the arterioles or by indirect effects. A redistribution of tumor blood flow was observed only after noradrenaline infusion and after vasopressin infusion. Noradrenaline was the only drug that enhanced the tumor blood flow in relation to surrounding parenchyma (liver).

## Future clinical aspects

In the present study a microsphere technique using a simultaneously drawn reference sample has been used in an experimental design in the anesthetized rat. The microsphere method cannot be used in its present form in humans as the microspheres are not degradable and might thus induce ischemic areas which, especially in the brain, might be deleterious. Degradable spheres with short degradation time could possibly be used in humans, but tissue biopsies have then to be removed before the degradation will take place. The  $^{99}\text{Tc}^{\text{m}}$  isotope seems to be ideal to use under these circumstances, with its short half life easily detectable  $\gamma$ -radiation of low energy. This isotope is also accepted in other clinical and diagnostic situations.

In the present experimental study in rats a relative decrease of total tumor blood flow with increasing size of the tumor was shown. Heterogeneous blood flow within the tumor with superior blood flow in the periphery versus the center of the tumor was also apparent. These findings support the general view that if cytostatic treatment of tumors, especially liver tumors is to be successful, these tumors should be treated when they are relatively small and when the circulation of the tumor is more homogeneous, and the tumors well vascularized. This means that the cytostatic drug more effectively reaches the target tissue. The tumor mass is also better controlled under these circumstances when the proliferative part of the tumor is relatively greater.

The use of a vasoactive drug as an adjuvant to regional cytostatic treatment of liver tumors might be of value in order to increase the local concentration. This has also been supported by findings by Iwaki et al (1978) who found that the addition of a catecholamine to mitomycin-C caused an increased uptake of the cytostatic drug in a rabbit liver tumor compared with surrounding liver tissue. The present study implies that noradrenaline at least in the rat, is possibly the most effective drug to enhance the relative blood flow of hepatic tumors. The other drugs tested in this series of experiments could not enhance the effects on the tumor blood flow which, however, does not exclude them from the therapeutic arsenal. Vasopressin might be used to induce ischemia in a tumor which then can be treated by for example hyperthermia. This has been proved to be most effective in ischemic areas like the center of tumors where the temperature rises to levels, lethal for the malignant cells. Radiation therapy, on the other hand, gives better results in well-oxygenized tissue and the vasoactive drugs tested in this study seem to have no effect in this aspect.

Transferred to the human situation it seems that noradrenaline and cytostatic drugs might be effectively infused into the hepatic artery using techniques that are readily available. This procedure should theoretically increase the relative uptake of a cytostatic drug in the tumor with less toxic effects on normal liver parenchyma and possibly also in the rest of the body. A vast field of experimental and clinical work is open for research. In the future the addition of a vasoactive drug to potent cytostatic drugs might bring relief to the patients suffering from cancer disease of the liver.



## SUMMARY

The purpose of the present investigation was to establish a method for simultaneous cardiac output determination and to study regional blood flow using microspheres labelled with radioactive isotopes. This was made possible by using the reference organ technique, which was relatively simple to handle both theoretically and in practice when the initial difficulties had been overcome. The results gave good reproducibility. The microsphere technique for blood flow measurements seems to be as good as any other method but has the advantage of allowing regional blood flow measurements and repeated studies in the same animal. Blood flow in malignant tumors inoculated in the liver and subcutaneously were studied by this technique and the general finding was that tumor blood flow was heterogenic and greater in the periphery of the tumor than in its center. Tumor blood flow was also found to be inversely related to tumor size.

Cardiovascular response and blood flow in normal tissue and tumors were also studied after infusion of five different vasoactive drugs with the same microsphere method but with microspheres labelled with one of two isotopes injected before and after infusion of the drugs. Thereby the rats can be used as their own controls in the statistical analysis. Cardiac output was significantly lowered by noradrenaline and vasopressin infusion and most tissues showed a decrease in blood flow after infusion of noradrenaline, vasopressin and adrenaline. Glucagon increased splanchnic blood flow but left hepatic arterial blood flow unchanged. Tumor blood flow was studied by establishing the ratio between tumor and liver blood flow and this ratio was significantly increased after infusion of noradrenaline and decreased after infusion of vasopressin. In subcutaneous tumors the ratio tumor to muscle or to cutaneous blood flow was studied and a significant decrease was found after infusion of noradrenaline, but no change was noted after infusion of any of the other drugs.

These findings indicate that only noradrenaline administered in intravenous infusion in anesthetized rats can enhance tumor blood flow and thus act as an adjuvant in the treatment of liver malignancy in combination with cytostatic drugs. This has to be further investigated in experimental models and in the clinic.

## ACKNOWLEDGEMENTS

This work was supported by grants from The Swedish Cancer Society, grant no 98-B76-12X, the Medical Faculty of the University of Lund, Sweden, John and Augusta Persson's Foundation for Scientific Medical Research, Lund, Sweden.

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